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Evaluation of impact of different antioxidants on stability of dietary folates during food sample preparation and storage of extracts prior to analysis

Johan D.M. Patring *, Madelene S. Johansson, Elena Yazynina, Jelena A. Jastrebova

Department of Food Science, Division of Food Chemistry, Swedish University of Agricultural Sciences, P.O. Box 7051, SE-750 07 Uppsala, Sweden

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Abstract

A troublesome factor during folate analysis is the instability of folates, especially tetrahydrofolate. This investigation was therefore performed to evaluate the relative effectiveness of four antioxidants (2-mercaptoethanol, dithiothreitol, 2,3-dimercapto-1-propanol, and 2-thiobarbituric acid) as stabilizing agents. The antioxidants were tested in the combination with 2% sodium ascorbate in acetate, phosphate, and HEPES/CHES buffers commonly used to extract folates from food samples. Baker's yeast was chosen as a model food matrix. A validated method based on reversed-phase high performance liquid chromatography with fluorescence and diode array detection was used for folate analysis. We showed that sample handling was of critical importance in folate analysis. Heat treatment, long-term storage, and repeated freeze/thaw cycles could impair the stability of tetrahydrofolate in varying degrees depending on buffers and antioxidants used. 2,3-Dimercapto-1-propanol was better than 2-mercaptoethanol in protecting tetrahydrofolate under heat extraction, long-term storage of food extracts and freezing/thawing. The use of 2,3-dimercapto-1-propanol as stabilizing agent in folate analysis may, therefore, be preferable owing to its lower toxicity and higher protective effectiveness. Preparation of food samples should include as few freeze/thaw steps as possible before analysis to prevent degradation of tetrahydrofolate.

Keywords: Folate analysis; Stability; Sample pre-treatment; Storage; Freezing/thawing; Antioxidant

1. Introduction

Folates, a group of water soluble B-vitamins, have received much attention due to their health promoting effects, especially because they reduce the risk of neural tube defects of new-born children [1–3] and prevent cardiovascular disease and certain forms of cancers [4,5]. Folates are required for central cellular functions such as amino acid biosynthesis, replication, and growth [4,6]. Determination of folates is therefore important in order to accurately establish actual folate intake in a population, where many people do not reach the recommended daily intake (400 μg folates for adults, 600 μg for pregnant women, and 500 μg for lactating women) [7,8].

Several analytical techniques have been used over the years to study the folate content in foods [9–12]. However, the analysis of food samples is complicated due to the diversity and instability of dietary folates and complexity of food matrixs [13]. Folates are sensitive to degradation induced by heat, UV light, and oxygen [10,14]. The stabilization of folates during the sample preparation, storage, and analysis is therefore important. Many studies have investigated the relative stability of different folate forms in buffered solutions as a function of pH, oxygen concentration, and temperature [15–24], but the stability of folates in food extracts prior to analysis is less well understood [13].

Today, to minimize degradation of folates induced by oxygen and UV light, the use of subdued light and flushing with nitrogen is recommended [9,16]. However, these precautions are not sufficient to prevent all losses of folates due to breakdown. The use of antioxidants, such as ascorbic acid and 2-mercaptoethanol (MCE), is thus essential for stabilization of folates and greater accuracy in folate analysis [15,16,25,26]. As shown by Wilson and Horne [26] the combination of 2-mercaptoethanol (MCE) and sodium ascorbate provided better stabilization of folates than sodium ascorbate alone. The authors reported that during heat extraction formaldehyde was formed from ascorbate anion and caused interconversion of the various reduced folate forms

* Corresponding author. Tel.: +46 18 672070; fax: +46 18 672995.
E-mail address: johan.patring@lmv.slu.se (J.D.M. Patring).

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[25]. This adverse effect could be totally eliminated by use of both sodium ascorbate (2%) and MCE (0.2 M) in HEPES/CHES buffer, pH 7.85 [26], commonly referred to as the Wilson and Horne extraction buffer. Similar results about the benefits of combining two antioxidants (ascorbic acid and MCE) have also been reported by Vahteristo et al. [27] and Pfeiffer et al. [28] when using phosphate buffer and HEPES/CHES buffer, respectively.

The combination of two antioxidants, ascorbic acid and MCE, is today, by far, the most commonly used approach to stabilize folates during sample preparation and analysis [13]. However, in the literature, we have not been able to find any comparative study of antioxidants, which has investigated alternatives to MCE with respect to their effectiveness as protecting agents for folates. The use of MCE is in fact undesirable for toxicological and ecological reasons (see Section 2.7). Finding a more effective, less toxic agent is therefore of interest and was one of the main purposes of this study. Furthermore, we have not been able to find any extensive study that in detail has investigated how storage and freezing/thawing affect folate stability in food extracts. There are today no official guidelines on how to store and treat an extracted food sample containing folates, which can lead to errors in inter-comparative studies. We wanted therefore to study how sample pre-treatment influenced the folate stability in extracted samples and to suggest a suitable way of acting to prevent significant folate losses due to degradation.

To do this, we first did a comparative study to evaluate the protecting effectiveness of different antioxidants to be used in combination with sodium ascorbate. Antioxidants used were MCE, dithiothreitol (DTT), 2,3-dimercapto-1-propanol (BAL), and 2-thiobarbituric acid (TBA). From this study, two antioxidants were chosen for an extended stability study of folates under different treatment conditions in extracts of baker's yeast. A validated method based on reversed-phase high performance liquid chromatography (HPLC) with fluorescence and diode array (DAD) detection was used for folate analysis.

2. Experimental

2.1. Reagents

Acetonitrile was of isocratic grade for HPLC; the other chemicals were of analytical quality. If not otherwise stated, the chemicals were purchased from Merck (Darmstadt, Germany). DL-Dithiothreitol, 2,3-dimercapto-1-propanol, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) were obtained from Sigma–Aldrich (St. Louis, USA). Water was purified using a Milli-Q system (Millipore, USA). Rat serum was obtained from Scanbur (Sollentuna, Sweden). Dry baker's yeast, *Saccharomyces cerevisae* (trademark: original kronjäst) was a gift from Jästbolaget, a Swedish yeast company in Rotebro, Sweden. It was vacuum packed in plastic bags immediately after delivery and stored frozen at −80 °C until analysis.

(6S)-5,6,7,8-tetrahydrofolate, sodium salt (H_4folate) and (6S)-5-methyl-5,6,7,8-tetrahydrofolate, sodium salt (5-CH_3-H_4folate) were a kind gift from Merck Eprova AG, Schaffhausen, Switzerland. The folate standards were stored at −80 °C until use. The purity of all standards was checked according to the procedure of van den Berg et al. [29] using molar extinction coefficients reported by Eitenmiller and Landen [9]. The standard stock solutions of folates of 200 μg/mL (purity corrected) were prepared under subdued light in 0.1 M phosphate buffer pH 6.1 containing 1% sodium ascorbate (w/v) and 0.1% MCE (v/v). Aliquots of the standard stock solutions were placed in separate tubes, flushed with nitrogen and stored below −80 °C at most 3 months. The calibration solutions were prepared immediately before use by dilution of the stock solution with extraction buffer (0.1 M phosphate buffer pH 6.1 with 2% sodium ascorbate (w/v) and 0.1% MCE (v/v)).

2.2. Stabilization of H_4folate in different buffer solutions

Four antioxidants (DTT, BAL, TBA, and MCE) were tested in different buffer solutions, all containing 2% sodium ascorbate, to evaluate their effectiveness to stabilize H_4folate (the most labile folate form) during boiling at different pH values. The buffers involved were 0.1 M sodium acetate buffer pH 5.0; 0.1 M phosphate buffer pH 6.1 and 0.1 M HEPES/CHES buffer pH 7.8. Each antioxidant was used in the concentration of 0.1% (v/v or w/v) for each buffer, which were all spiked with 100 ng/mL of H_4folate. The prepared volume of each buffer solution being tested was 25 mL. From this solution 10 mL (duplicates) were transferred to glass tubes, which were flushed with nitrogen for 15 s, capped, and placed in a boiling water bath for 1 h. Thereafter, they were cooled on ice and then transferred to vials and placed directly in the thermostated autosampler for analysis on HPLC. The obtained concentrations of H_4folate were compared to the concentrations of H_4folate in the remaining unboiled samples (5 mL) in the respective buffer solutions for each antioxidant. These unboiled samples were placed in the thermostated autosampler directly after the preparations, and the analysis began immediately.

2.3. Stabilization of H_4folate and 5-CH_3-H_4folate in baker's yeast extracts

The effects of freeze/thawing and storage of samples in freezer (−22 °C) were evaluated for the stability of folates in extracts of baker's yeast. Comparison was also made of the relative effectiveness between MCE and BAL. For this purpose, two extraction buffers were compared: 0.1 M phosphate buffer pH 6.1 with 2% sodium ascorbate (w/v) and 0.1% MCE (v/v) (MCE-buffer) and 0.1 M phosphate buffer pH 6.1 with 2% sodium ascorbate (w/v) and 0.1% BAL (v/v) (BAL-buffer).

Extraction of folates from baker's yeast was performed by dissolving 50 mg of yeast in 20 mL extraction buffer (MCE- or BAL-buffer) and boiling for 12 min as described in a validated method for baker's yeast [30]. Deconjugation of folate polyglutamates to monoglutamates was done by adding 50 μL of rat serum containing folate conjugase to 1 mL of the yeast extract in a glass tube and incubation in a shaking water bath at 37 °C for 3 h. The obtained yeast extracts containing folate monoglutamates were analyzed by HPLC.

Table 1
Description of the different sample pre-treatments of yeast extracts

A	C	B	D	E
Extraction		Extraction		Extraction
↓		↓		↓
Deconjugation		Freezing (18 h)		Freezing (4 weeks)
(Analysis of unstored samples)		↓		↓
↓		Thawing (once)		Thawing (once)
Freezing (1, 2, 3, or 4 weeks)		↓		↓
↓	↓	Deconjugation		Deconjugation
Thawing (once)	Repeated	(Analysis of unstored samples)		↓
↓	thawing/freezing *	↓		Analysis
Analysis	(up to 4 times)	Freezing (1, 2, 3, or 4 weeks)		
	↓	↓	↓	
	Analysis	Thawing (once)	Repeated	
		↓	thawing/freezing *	
		Analysis	(up to 4 times)	
			↓	
			Analysis	

* the same single yeast extract was thawed/frozen every seventh day during four weeks.

Five different sample treatments were tested for each antioxidant (Table 1). Each treatment was performed in triplicates. The treatment A included extraction and deconjugation of folates on the same day. The treatment B involved extraction of folates with an intermediate freezing step (18 h) before the deconjugation. For both treatments, aliquots of yeast extract were placed in 12 vials, respectively, and frozen at −22 °C. Every seventh day for 4 weeks, three vials were thawed and the folate content was analyzed by using HPLC. The obtained folate concentrations were compared to those obtained on the first sample pre-treatment day (treatment A–D, unstored, Table 1) to evaluate an eventual degradation. The treatments C and D were done exactly as treatment A and B, respectively, but the same single yeast extract was thawed/frozen every seventh day to evaluate the folates' sensitivity to repeated freezing/thawing. For the treatment E, the extracted yeast samples were stored non-deconjugated in freezer (−22 °C) for 4 weeks and were then thawed and immediately deconjugated and analyzed on HPLC. This means that freezing/thawing was only performed once and could therefore be compared with treatment A, i.e. when stored for 4 weeks (see Table 1).

2.4. Chromatographic equipment and conditions

Analyses were performed using an HPLC system (Agilent 1100) consisting of a gradient quaternary pump, a thermostated autosampler, a thermostated column compartment, a diode array detector (DAD) and a fluorescence detector. The HPLC system was controlled by a personal computer running Agilent Chemstation software. The separation of folates was performed on an Aquasil C_{18} column, 150 mm × 4.6 mm; 3 μm (Thermo Electron Corporation, USA) with a guard column Opti-guard C_{18}, 1 mm (Optimize Technologies Inc., USA) at 23 °C. The flow rate was 0.4 mL/min; the injection volume 20 μL; the temperature in the thermostated autosampler 8 °C. For the detection and quantification of H_4folate and 5-CH_3-H_4folate a fluorescence detector was used (excitation at 290 nm and emission at 360 nm). The mobile phase used was acetonitrile-30 mM phosphate buffer (pH 2.3) under linear gradient elution conditions. The gradient started at 6% (v/v) acetonitrile with a lag of 5 min, then the gradient was raised linearly to 25% acetonitrile during 20 min and was kept constant for 2 min; thereafter, it was decreased linearly to 6% acetonitrile during 1 min and was applied for 14 min to re-equilibrate the column. Retention times of folate standards were used for peak identification; comparison of ratio of sample peaks from fluorescence and diode array detectors to ratio of standard peaks as well as fluorescence and diode array spectra were used to verify peaks if necessary.

2.5. Quantification

Quantification was based on an external standard method in which the peak area was plotted against concentration. A multilevel calibration curve was used ($n = 7$) and least-squares regression analysis was used to fit lines to the data. The amount of each folate form was calculated in its free acid form.

Table 2
Safety considerations for antioxidants used in the study

Antioxidant	Indication of danger	Risk statements
MCE	Toxic (EU)[a]; highly toxic (USA)[b]; dangerous for the environment	Harmful if swallowed; toxic in contact with skin; causes burns; toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environments
BAL and DTT	Harmful (EU)[a]; toxic (USA)[b]	Harmful if swallowed; irritating to eyes, respiratory system and skin
TBA	No indication of danger	No risk statements

[a] The European Union regulatory information.
[b] The United States regulatory information.

2.6. Statistics

For stability study in buffer solutions all results were presented as means of duplicates, the difference between two separate values was less than 10% for all samples. For stability study of folates in yeast all results were presented as means of triplicates; the relative standard deviation was less than 7% for all samples.

Two-way ANOVA with interaction was calculated to see possible significant differences in the degradation of H_4folate after boiling due to the antioxidant or buffer composition. Two-way ANOVA with interaction for each antioxidant, MCE and BAL, was also calculated for each folate form found in baker's yeast to determine whether significant main effects could be shown due to sample pre-treatment and storage time. In addition, two-way ANOVA with interaction for each sample pre-treatment (in total four) were calculated to compare the relative effectiveness between MCE and BAL. If significant differences could be shown in ANOVA analysis, Duncan's multiple range test was used to pinpoint where these differences occurred. To compare treatment A (i.e. samples stored for 4 weeks) with treatment E, two-sided *t*-test of the mean was calculated for both MCE and BAL. A *p*-value <0.05 was considered significant for the ANOVA analyses, Duncan's multiple range test and the *t*-test. All statistical analyses were performed as described by Miller and Miller [31] and Milton [32] by use of Microsoft Office Excel 2003.

2.7. Safety consideration

General guidelines for work with organic solvents and acids were respected. Safety considerations for antioxidants used in the study are presented in Table 2.

3. Results and discussions

3.1. Stabilization of H_4folate in buffer solutions

The choice of buffer in folate analysis often depends on the pH optima of the enzymes used during the deconjugation of folate polyglutamates to monoglutamates, i.e. after the extraction step [9,11,33]. The most commonly used buffers are acetate, phosphate, and HEPES/CHES buffer [9,13]. These three buffers were therefore selected for our comparative study to prepare solutions containing H_4folate in the presence of antioxidants. Boiling in a water bath was chosen as a simple sample treatment in order to compare the effectiveness of different antioxidants since folates, especially H_4folate, are sensitive to heat.

Table 3
Comparison of the protective effectiveness of antioxidants for H_4folate in different buffers during boiling (1 h)

Buffer	Antioxidant + 2% sodium ascorbate
Acetate (pH 5.0)	DTT (100) ≫ BAL (96) = TBA (95) ≫ MCE (88)
Phosphate (pH 6.1)	BAL (100) = DTT (100) ≫ TBA (96) > MCE (91)
HEPES/CHES (pH 7.5)	TBA (100) ≫ DTT (96) = MCE (93); TBA = BAL (98); BAL = DTT; BAL ≫ MCE

>, significantly more effective antioxidant with $p < 0.05$; ≫, significantly more effective antioxidant with $p < 0.01$; =, no significant difference in protective effectiveness ($p \geq 0.05$). The numbers in brackets are the percentage of H_4folate remaining after 1 h boiling as compared to the unboiled samples. Values are means of duplicates. For statistics see Section 2.

The stabilizing effect of antioxidants in the different buffer solutions expressed as percentage H_4folate left after 1 h boiling when compared to the unboiled samples (100%), can be seen in Table 3. The effectiveness of each single antioxidant to protect H_4folate did not depend significantly on the buffer solution used, despite some small variations. As the data of Table 3 suggest, there were no significant interactions ($p \geq 0.05$) and no significant differences due to buffer solution ($p \geq 0.05$). However, the degree of effectiveness in protecting H_4folate from degradation in a given buffer solution depended significantly ($p < 0.05$) on the antioxidant used (Table 3). DTT was the most effective antioxidant in acetate buffer; DTT and BAL, in phosphate buffer; and BAL and TBA, in HEPES/CHES, whereas MCE was the least effective in all buffer solutions compared to the other antioxidants (degradation range 7–12%). Our findings were in good agreement with an earlier study of the stability of H_4folate, dissolved in 0.1 M phosphate buffer, pH 7.2 [34]. As shown by Blakley, there was a rapid degradation of H_4folate without antioxidants, but the use of BAL in 1 mM concentration (0.01%, v/v) could prevent degradation for several hours, whereas the use of MCE in 5 mM concentration (0.035%, v/v) was much less effective, with an immediate degradation of H_4folate.

3.2. Stability studies of folates in real food samples

When analyzing folates in real foods, sample preparation includes both heat extraction at 70–121 °C (5–15 min) and mono-, di- or tri-enzyme treatment at 37 °C during 3–30 h [9,12,13,28]. Moreover, food extracts are often stored frozen prior to analysis and may be analyzed at several occasions [28,35–38]. Sometimes sample pre-treatment also involves intermediate freezing of food homogenates prior to extraction step [39,40] and/or freezing of prepared food extracts before enzyme treatment [41,42]. It was therefore of interest to evaluate how different steps of sample pre-treatment, including storage and freezing/thawing of samples, affects the folate stability in real food extracts. For this test baker's yeast was chosen as a

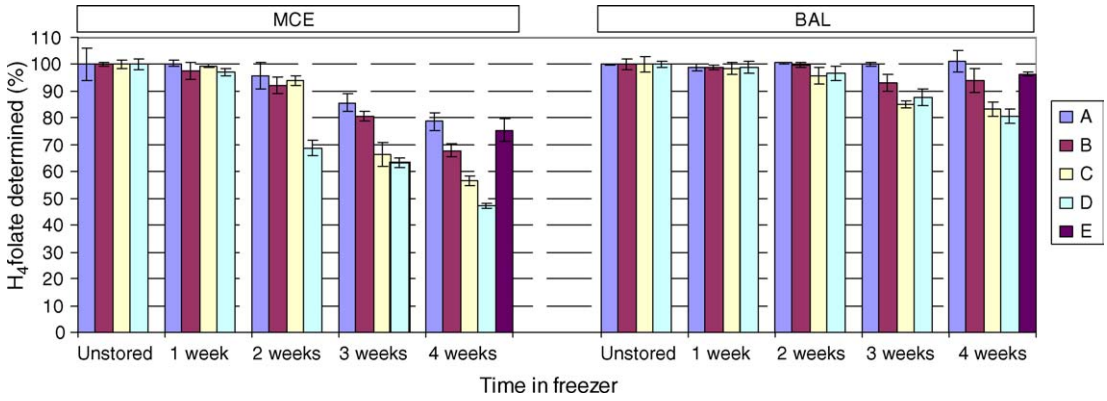

Fig. 1. The stability of H_4folate in yeast extracts expressed as percentage H_4folate remaining after treatment as compared to the unstored samples. For extraction 0.1 M phosphate buffer pH 6.1 containing 2% sodium ascorbate (w/v) was used with either 0.1% MCE (v/v) or 0.1% BAL (v/v). All values are means of triplicates; for the explanation of treatments (A–E), see Table 1.

model food matrix since it contains considerable amounts of two main folate forms (5-CH_3-H_4folate and H_4folate) and can be analyzed by a simple and reliable HPLC method (recovery 97–98% and intra- and inter-day-precision 1.9–4.0 (R.S.D.%) for the different folate forms) [30]. Phosphate buffer pH 6.1 was used as extraction buffer because the folate conjugase in rat plasma/serum has been shown to be most effective at pH around 6 [43]. As indicated in the present study, BAL and DTT were the most effective antioxidants to protect H_4folate in phosphate buffer during boiling. BAL was chosen for stabilization of folates in yeast samples because it is a liquid and is therefore easier to handle than DTT. Since MCE is by far the most commonly used antioxidant in folate analysis, it was also chosen, despite an apparent lower efficiency in stabilization of H_4folate during boiling (Table 3).

The stability of H_4folate and 5-CH_3-H_4folate in yeast extracts over time when using MCE and BAL as antioxidants can be seen in Figs. 1 and 2, respectively. Evidently the stability of these two folate forms differed considerably. 5-CH_3-H_4folate appeared to be stable under the test conditions; no significant differences were found ($p \geq 0.05$) due to treatment, storage time or antioxidant used. This confirmed earlier findings that 5-CH_3-H_4folate is more stable and less sensitive to degradation as compared to H_4folate, as reviewed by Gregory [14]. In contrast

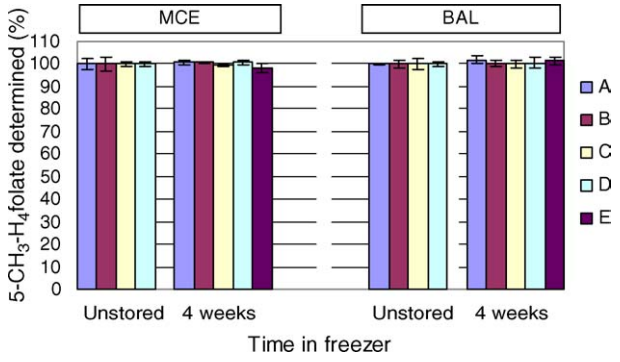

Fig. 2. The stability of 5-CH_3-H_4folate in yeast extracts expressed as percentage 5-CH_3-H_4folate remaining after treatment as compared to the unstored samples. For extraction 0.1 M phosphate buffer pH 6.1 containing 2% sodium ascorbate (w/v) was used with either 0.1% MCE (v/v) or 0.1% BAL (v/v). All values are means of triplicates; for the explanation of treatments (A–E), see Table 1.

to 5-CH_3-H_4folate, H_4folate degraded considerably during long-term storage of yeast extracts. The losses of H_4folate ranged up to 53% after 4 weeks of storage at −22 °C (Fig. 1). ANOVA calculations showed significant differences ($p < 0.001$) between storage times for both antioxidants (Table 4). These results were in good agreement with earlier investigations of H_4folate stability. As shown by Tamura et al. [39], storage at −70 °C for a few months caused considerable degradation of H_4folate (approximately 60%) in food extracts.

As seen from Table 5, long-term stability of H_4folate during storage of yeast extracts in freezer differed significantly when using BAL and MCE. Obviously BAL over time was a much more effective protecting agent as compared to MCE. With BAL,

Table 4
Influence of different storage times of yeast extracts in freezer on stability of H_4folate

Treatment	Antioxidant	
	MCE	BAL
A	0 = 1 ≫ 2 ≫ 3 ≫ 4	0 = 1 = 2 = 3 = 4
B	0 = 1 ≫ 2 ≫ 3 ≫ 4	0 = 1 = 2 ≫ 3 = 4
C	0 = 1 ≫ 2 ≫ 3 ≫ 4	0 = 1 > 2 ≫ 3 = 4
D	0 = 1 ≫ 2 ≫ 3 ≫ 4	0 = 1 > 2 ≫ 3 ≫ 4

0, unstored samples; 1, stored at −22 °C for 1 week and so on; >, significantly better stability with $p < 0.05$; ≫, significantly better stability with $p < 0.01$; =, no significant difference ($p \geq 0.05$). For treatment explanation see Table 1; for statistics see Section 2.

Table 5
Comparison of the protective effectiveness of BAL and MCE for H_4folate in yeast extracts

Storage time	Treatment			
	A	B	C	D
Unstored	MCE = BAL	MCE = BAL	MCE = BAL	MCE = BAL
1 week	MCE = BAL	MCE = BAL	MCE = BAL	MCE = BAL
2 weeks	BAL ≫ MCE	BAL ≫ MCE	MCE = BAL	BAL ≫ MCE
3 weeks	BAL ≫ MCE	BAL ≫ MCE	BAL ≫ MCE	BAL ≫ MCE
4 weeks	BAL ≫ MCE	BAL ≫ MCE	BAL ≫ MCE	BAL ≫ MCE

≫, significantly more effective antioxidant ($p < 0.01$); =, no significant difference in protective effectiveness ($p \geq 0.05$). For treatment explanation see Table 1; for statistics see Section 2.

Table 6
Comparison of different sample pre-treatments (A–D) in respect to stability of H_4folate

Storage	Antioxidant	
	MCE	BAL
Unstored	A = B = C = D	A = B = C = D
1 week	A = B = C = D	A = B = C = D
2 weeks	A = B = C > D	A = B ≫ C = D
3 weeks	A ≫ B ≫ C = D	A ≫ B ≫ C = D
4 weeks	A ≫ B ≫ C ≫ D	A ≫ B ≫ C > D

>, significantly better stability with $p < 0.05$; ≫, significantly better stability with $p < 0.01$; =, no significant difference ($p \geq 0.05$). For treatment explanation see Table 1; for statistics see Section 2.

the losses of H_4folate due to degradation did not exceed 19%, whereas they reached 53% with MCE (Fig. 1). However, the differences between these two antioxidants depended greatly on the storage time in the freezer. During the first week of storage, no differences could be observed between BAL and MCE, but after 1 week of storage BAL was, regardless of treatment, a significantly better stabilizing agent for H_4folate than MCE, except for treatment C week 2, where no significant difference could be observed.

Repeated freezing/thawing of yeast extracts, especially third and fourth freeze/thaw cycles, were found to adversely affect the stability of H_4folate (see treatments C and D in Fig. 1). As seen from Table 6, the different treatments differed significantly (ANOVA; $p < 0.001$) for both BAL and MCE. Treatment A with no or single freeze/thaw cycle always had the least degradation of H_4folate, followed by treatments B–D. As for long-term storage, BAL appeared to stabilize H_4folate much better than MCE. After four freeze/thaw cycles (treatments C and D) the losses of H_4folate comprised 17–19% when using BAL compared to 43–53% with MCE.

An intermediate freeze/thaw step before deconjugation (treatments B and D) also impaired the stability of H_4folate. With this step the losses of H_4folate were significantly higher compared to treatments A and C not including intermediate freezing (Fig. 1, Table 6). Moreover, long-term storage of yeast extracts (4 weeks, treatment E) prior to deconjugation step resulted in further increase of degradation of H_4folate compared to short-term intermediate freezing (18 h, treatment B, unstored) as seen in Fig. 1.

Mono- and polyglutamates of H_4folate differed significantly regarding long-term stability in freezer when using both BAL ($p < 0.001$) and MCE ($p < 0.05$). As seen in Fig. 1, H_4folate monoglutamate in deconjugated yeast extracts (treatment A) was more stable than H_4folate polyglutamates in non-deconjugated yeast extracts (treatment E) on storage at −22 °C during 4 weeks. This means that deconjugation should be performed immediately after extraction without any intermediate freezing step to enhance the stability of H_4folate.

4. Conclusions

This is the first extensive study, to our knowledge, on the effects of different antioxidants, storage time, and freeze/thaw cycles on the stability of dietary folates in buffers and real food extracts. It was found that the degree of effectiveness in protecting H_4folate from degradation in a given buffer solution during boiling for 1 h depended on the type of antioxidant used. BAL was much better than MCE regarding protection of H_4folate under boiling for 1 h in phosphate buffer pH 6.1. Furthermore, when using baker's yeast as a model food matrix in phosphate buffer pH 6.1 and enzyme treatment with rat serum conjugase, BAL provided much better protection of H_4folate than MCE during long-term storage in freezer and thawing/freezing as well. The use of BAL as stabilizing agent in folate analysis may be preferable owing to its higher protective effectiveness in respect to H_4folate and lower toxicity in comparison with MCE. In contrast to H_4folate, 5-CH_3-H_4folate appeared to be stable under the conditions tested in this study, which was in good agreement with earlier studies.

The study showed that sample handling is of critical importance in folate analysis of foods. Heat treatment, long-term storage, and repeated freeze/thaw cycles could impair the stability of H_4folate in varying degrees depending on buffers and antioxidants used. To completely prevent degradation of H_4folate, sample preparation should include as few freeze/thaw steps as possible before analysis and storage time of food extracts needs to be kept to a minimum.

It should also be stressed that food components may influence the stability of folates, which can result in considerable variations of folate stability in different food matrices of even similar nature. For this reason, a check on folate stability seems to be necessary for each specific food matrix.

Acknowledgments

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